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Differential Regulation of Exocytosis by Calcium and CAPS in Semi-Intact Synaptosomes

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Summary

Using a novel approach to measure exocytosis in vitro from semi-intact synaptosomes, we establish that the Ca^{2+}-dependent release of glutamate requires cytosolic factors for mobilization from the reserve pool. The cytosolic activity for glutamate release was not satisfied by CAPS, a soluble component required for norepinephrine (NE) release. Moreover, the CAPS-independent glutamate release from synaptic vesicles (SVs) was 200-fold less sensitive to Ca^{2+} than that required for dense core vesicles (DCVs). The differential regulation of exocytosis by CAPS, Ca^{2+}, and potential novel cytosolic factor(s) suggests that the docking and fusion machinery controlling DCVs has diverged from that regulating glutamate-containing SVs.

Introduction

The entry of Ca^{2+} into nerve terminals initiates a series of molecular events that culminates with the fusion of synaptic vesicles and release of neurotransmitter into the synaptic cleft (reviewed by Calakos and Scheller, 1996). This process, which underlies the integrative function of the brain, involves a highly coordinated cascade of membranous and cytoplasmic proteins. Factors that regulate vesicle targeting and fusion have been defined not only on the basis of their cellular localization in the synapse but also on the basis of their biochemical interactions in vitro and in vivo. It is now recognized that many of the proteins that control vesicular traffic at the synapse, including VAMP/synaptobrevin, syntaxin 1, n-Sec1, SNAPS, and SNAP-25, as well as GTPases belonging to the Rab3 family, are evolutionarily conserved and fulfill similar functions at other steps of the endocytotic and exocytotic pathway (reviewed by Bajjalieh and Scheller, 1995; Linial, 1997). However, the physiological roles of these synaptic proteins remain elusive, in part owing to the inaccessibility of the secretion machinery to direct experimentation.

In recent years, the reconstitution of secretion from semi-intact PC12 cells has allowed the identification and purification of cytosolic factors essential for priming or fusion of dense-core vesicles (DCV) (Walent et al., 1992; Hay and Martin, 1993). A 145 kDa protein, CAPS (Ca^{2+}-activator protein for secretion), homologous to the *C. elegans* UNC-31 gene product, is now recognized as essential for norepinephrine (NE) release from PC12 cells. Although CAPS is widely expressed in neural and endocrine tissues that exhibit regulated secretion, and is particularly abundant in brain, its function in neurons has not been explored. Berwin et al. (1998 [this issue of *Neuron*]) have characterized the subcellular localization of CAPS in rat brain synaptosomes. The protein is preferentially associated with DCVs and apparently excluded from clear synaptic vesicles (SV). However, it remains unclear whether this differential localization on DCVs is related to an exclusive regulation of DCV exocytosis, and therefore whether the fusion of SVs occurs independently of CAPS.

To address whether CAPS is involved solely in the regulation of DCV- and not SV-mediated secretion from nerve terminals, we have developed an experimental system that employs semi-intact synaptosomes to study the fusion of vesicles containing glutamate or NE to the cell surface in a Ca^{2+}- and cytosol-dependent fashion. Transmitter release from this semi-intact preparation reconstitutes the phenotypic properties of exocytosis observed in vivo. We now demonstrate that CAPS is not required for exocytosis of glutamate-containing vesicles. These results have important implications with regard to the underlying common and distinct biochemical machineries controlling vesicle docking and fusion in nerve terminals.

Results

Cytosolic Factors Are Necessary for the Reconstitution of Transmitter Release from Semi-Intact Synaptosomes

To define the role of cytosolic CAPS in the regulation of neurotransmitter release, we established an assay that efficiently reconstitutes exocytosis from nerve terminals in vitro. The glutamate content of synaptosomes rendered "semi-intact" by exposure to an optimal concentration of streptolysin O (SLO) was reduced by 50%. The size of the remaining transmitter pool was resistant to repeated washing, with $<10\%$ lost during 2 hr storage on ice, thereby defining the compartmentalized pool. Examination of intact and SLO-permeabilized synaptosomes by electron microscopy revealed that numerous small clear vesicles of $\sim$45 nm diameter and occasional dense-core vesicles of $\sim$90 nm diameter dispersed throughout the cytoplasm. A small number of the clear vesicles ($<5\%$) were observed in close apposition to the synaptic membrane opposing a postsynaptic density. The major difference between intact and semi-intact synaptosomes was the less dense appearance of the cytoplasm, a consequence of the diffusion of soluble cytoplasmic proteins. Consistent with this, pore formation was accompanied with efflux of the soluble pools of Rab3A, NSF, and α/β-SNAPs. In contrast, the distribution of the integral membrane proteins VAMP/synaptobrevin and syntaxin remained unchanged following permeabilization, attesting to the retention of synaptic

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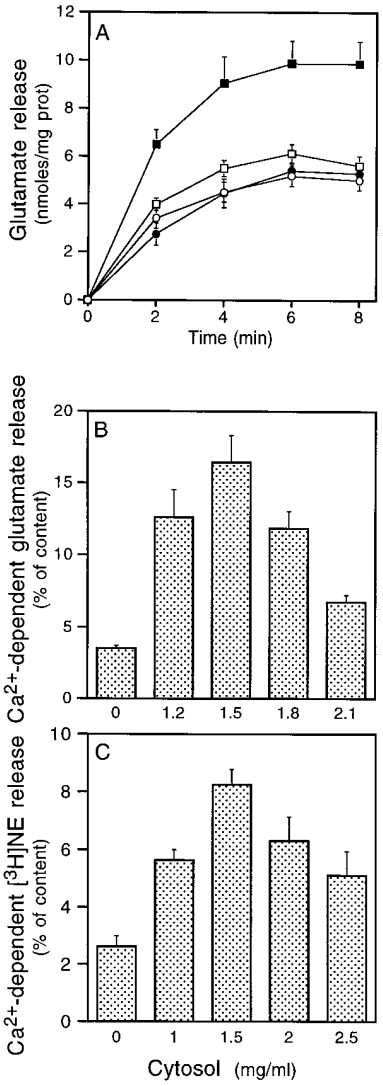

Figure 1. Ca^{2+}- and Cytosol-Dependent Glutamate Release Reconstituted from Semi-Intact Synaptosomes

(A) Time course of total glutamate release from semi-intact synaptosomes exposed to Ca^{2+}-free buffer (open circles), Ca^{2+}-free buffer with 1.5 mg/ml cytosol (closed circles), 200 μM Ca^{2+} buffer (open squares), or 200 μM Ca^{2+} buffer with 1.5 mg/ml cytosol (closed squares).

(B and C) Ca^{2+}-dependent component of glutamate (B) or [^{3}H]NE (C) release from semi-intact synaptosomes during a 4 min stimulation at 37°C with 200 μM Ca^{2+} buffer in the presence of indicated concentrations of cytosol. Panels show mean ± SEM from four to seven separate experiments.

vesicles and presynaptic membranes observed using electron microscopy (data not shown).

Incubation of the semi-intact synaptosomes at 37°C in the presence of Ca^{2+} and cytosol led to the release, on average, of ~45% of the total compartmentalized glutamate content (20–25 nmol/mg protein) in different preparations. Secretion consisted of a Ca^{2+}-independent pool (15%–20% or 4–5 nmol/mg protein; Figure 1A, open circles) that could not be stimulated further with cytosol (Figure 1A, closed circles) and was attributed

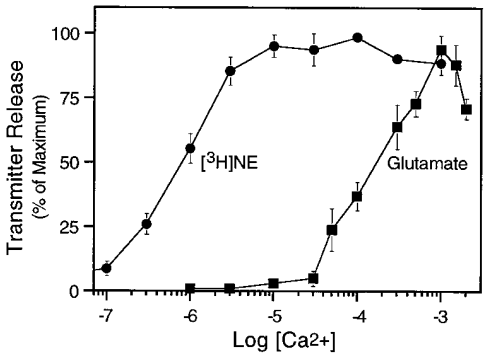

Figure 2. Differential Ca^{2+} Sensitivity of Glutamate and [^{3}H]NE Release

Glutamate (closed squares) and [^{3}H]NE (closed circles) release triggered by indicated concentrations of Ca^{2+} during 10 min stimulation at 25°C in the presence of 1.5 mg/ml cytosol. The optimal release in individual experiments (n = 4) was considered maximum.

to temperature-induced leakage from vesicles. Supplementation with 200 μM Ca^{2+} alone (Figure 1A, open squares) released an additional increment of ~5%, whereas the inclusion of cytosol with Ca^{2+} (Figure 1A, closed squares) triggered the release of another 20% of the glutamate stores, more than double the Ca^{2+}-independent background. The cytosol-stimulated glutamate release in the presence of Ca^{2+} was saturable (Figure 1B), being suboptimal at high cytosol concentrations possibly owing to an imbalance of components (Walent et al., 1992; Elazar et al., 1994). Semi-intact synaptosomes preloaded with [^{3}H]NE also released ~3% of their total [^{3}H]NE in response to the Ca^{2+} trigger, with cytosol stimulating up to 4-fold the Ca^{2+}-dependent signal (Figure 1C). Boiling or proteolysis of cytosol inactivated the cytosol-dependent secretion, demonstrating that the active component is a protein(s). Thus, Ca^{2+}-dependent glutamate and NE release from semi-intact synaptosomes can be distinguished from the Ca^{2+}-independent pool by its stimulation with cytosol.

Differential Sensitivity of Exocytosis to Ca^{2+}

As a test for the potential differential regulation of glutamate-containing SVs compared to DCVs, we determined the optimal Ca^{2+} concentration required to trigger release of each class. Glutamate and [^{3}H]NE release from semi-intact synaptosomes was measured in the presence of 1.5 mg/ml cytosol and Ca^{2+} buffers giving varying concentrations of free Ca^{2+}. The threshold for activation of glutamate release by Ca^{2+} was ~50 μM, with maximal release occurring at 1 mM (Figure 2, squares) and half-maximal release occurring at 200–300 μM Ca^{2+}. In striking contrast, the sensitivity of [^{3}H]NE release to the Ca^{2+} trigger was significantly higher, with an effective Ca^{2+} concentration range of 0.3–30 μM and half-maximal release evoked by 1 μM Ca^{2+}. The difference in the sensitivity to Ca^{2+} between glutamate and [^{3}H]NE release measured simultaneously in vitro represents more than two orders of magnitude and implies that the modulation of [^{3}H]NE exocytosis is likely to be distinct from that of glutamate exocytosis. To assess the ultrastructural changes that occur during transmitter release,

semi-intact synaptosomes were incubated with cytosol in the absence or presence of Ca^{2+} for 4 min and rapidly fixed for electron microscopy. This stimulation induced a dramatic change in the number of vesicles detectable in the nerve terminal. Using quantitative stereology, we found a 51% (± 5%) reduction in the overall number of vesicle profiles.

Ca^{2+}- and Cytosol-Dependent Release of Transmitter Reflects SNARE-Dependent Fusion of Vesicles

Clostridial neurotoxins exert a potent inhibitory action on the exocytotic machinery in nerve terminals in vivo by specific proteolysis of proteins involved in docking and fusion (reviewed by Niemann et al., 1994). To test whether the transmitter release from permeabilized synaptosomes faithfully reconstituted these events, synaptosomes were incubated with either botulinum neurotoxin B (Bot B) or E (Bot E), which specifically cleave synaptobrevin and SNAP-25, respectively (Schiavo et al., 1992; Schiavo et al., 1993; Binz et al., 1994). Prior to use, the holotoxins were treated with 1 mM dithiothreitol (DTT) for 30 min to ensure separation of the light and heavy polypeptides (Ahnert-Hilger et al., 1989; Stecher et al., 1989). In the absence of an associated heavy chain, the light chain, which contains the zinc-protease activity, requires direct access to the exocytotic apparatus (reviewed by Montecucco and Schiavo, 1993). Bot B eliminated virtually all of the Ca^{2+}-dependent release of glutamate, whereas Bot E reduced the signal by 60% (data not shown). The inhibition by the clostridial neurotoxins, combined with the observed decrease in the number of synaptic vesicles following Ca^{2+} exposure, demonstrates that the Ca^{2+}- and cytosol-dependent components of transmitter release are the consequence of the fusion of synaptic vesicles with the presynaptic membrane. Hence, the stimulation of semi-intact synaptosomes with Ca^{2+} in the presence of cytosol preserves the normal mechanism of glutamate vesicle exocytosis observed in intact nerve terminals.

CAPS Antibody Does Not Interfere with Glutamate Release

In PC12 cells, CAPS is an essential cytosolic factor that is required for reconstitution of Ca^{2+}-dependent [^{3}H]NE release (Walent et al., 1992; Martin and Kowalchyk, 1997). It is present specifically in cytosol derived from tissues that exhibit regulated secretion, suggesting a role in exocytosis, and is particularly abundant in rat brain cytosol, where it constitutes nearly 0.3% of cytosolic protein. Its potential role in regulating transmitter release from nerve terminals has not been investigated.

Semi-intact synaptosomes and cytosol were separately preincubated on ice with affinity-purified polyclonal CAPS antibody and then incubated at 25°C in the presence of Ca^{2+} and cytosol. The extent of [^{3}H]NE release from the antibody-treated synaptosomes was reduced by nearly 60% (Figure 3A). In contrast to the effects of the CAPS antibody on NE release, the extent of Ca^{2+}-dependent glutamate release was not affected by the antibody; both the kinetics and the extent of release were virtually indistinguishable between the control and antibody-treated synaptosomes (Figure 3B).

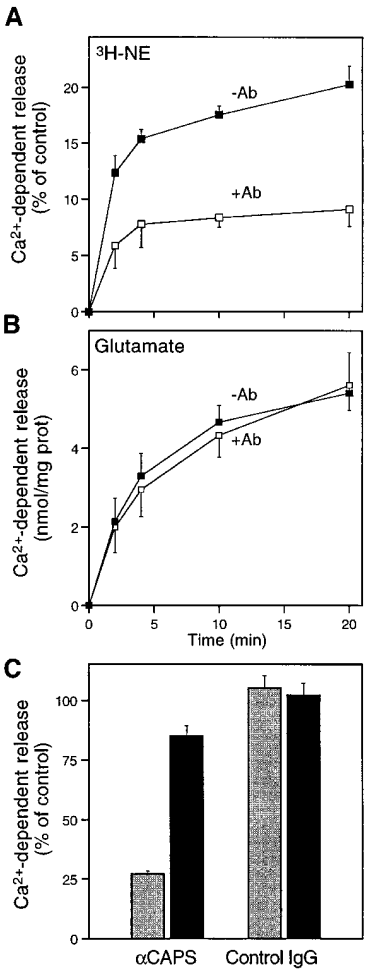

Figure 3. Effect of Anti-CAPS Antibody on [^{3}H]NE and Glutamate Release from Semi-Intact Synaptosomes

(A and B) Time-course of [^{3}H]NE (A) or glutamate (B) release triggered by 400 μM Ca^{2+} and 1.5 mg/ml cytosol at 25°C. Semi-intact synaptosomes and cytosol had been separately pretreated on ice for 2 hr with (open squares) or without (closed squares) 100 μg/ml anti-CAPS antibody before stimulation. Results show mean ± SEM from four separate experiments and are reported as Ca^{2+}-dependent signal only.

(C, αCAPS) Ca^{2+}-dependent release of [^{3}H]NE (shaded bar) and glutamate (solid bar) during 10 min stimulation with 30 μM Ca^{2+} or 400 μM Ca^{2+} (EC_{90} Ca^{2+} concentrations) and 1.5 mg/ml cytosol at 25°C. Semi-intact synaptosomes and cytosol were separately treated on ice for 2 hr with 400 μg/ml CAPS antibody prior to stimulation. This batch of antibody was different than that used in experiments described in (A) and (B). Results are expressed as a percent of Ca^{2+}-dependent release measured in the absence of antibody.

(C, Control IgG) Ca^{2+}-dependent release of [^{3}H]NE (shaded bar) and glutamate (solid bar) during 10 min stimulation with 400 μM Ca^{2+} and 1.5 mg/ml cytosol at 25°C. In these experiments, semi-intact synaptosomes and cytosol were separately treated on ice for 2 hr with 400 μg/ml control IgG prior to stimulation.

A second polyclonal CAPS antibody, previously shown to be inhibitory in PC12 cells (Martin and Kowalchyk, 1997), reduced [^{3}H]NE release by 73%—essentially all of the cytosol-dependent [^{3}H]NE secretion—without any significant effect on glutamate release (Figure 3C). Since treatment of synaptosomes and cytosol with control IgG

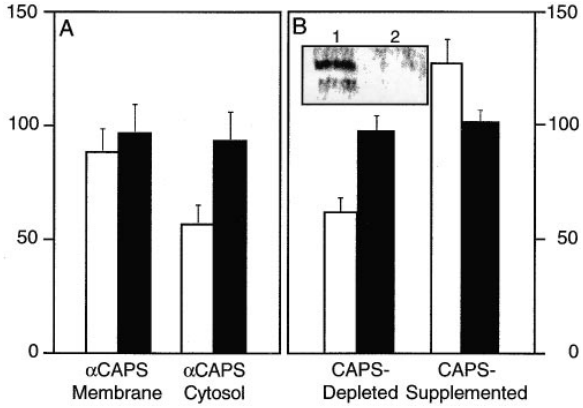

Figure 4. Recruitment of CAPS from Cytosol Is Essential for Optimal [^{3}H]NE Secretion but Not for Glutamate

(A) Semi-intact synaptosomes (membrane) or cytosol (cytosol) were separately pretreated with 100 μg/ml anti-CAPS antibody for 2 hr on ice. These aliquots were then added to reaction buffer containing untreated cytosol or semi-intact synaptosomes, respectively, as well as 400 μM Ca^{2+}. Bars depict Ca^{2+}-dependent (mean ± SEM of four individual experiments) release of [^{3}H]NE (open bars) and glutamate (solid bars) shown as a percent of the Ca^{2+}-dependent secretion from untreated synaptosomes and cytosol.

(B) Ca^{2+}-dependent [^{3}H]NE (open bar) and glutamate release (solid bar) in the presence of either CAPS-depleted cytosol or CAPS-depleted cytosol supplemented with recombinant CAPS. Bars represent Ca^{2+}-dependent release of transmitter (mean ± SEM of four individual experiments) shown as a percent of release in the presence of control cytosol.

(Inset) Western blot for CAPS in control cytosol (lane 1) and cytosol immunodepleted of CAPS (lane 2).

did not affect the release of glutamate or [^{3}H]NE (Figure 3C), it is apparent that glutamate release is insensitive to the previously established inhibitory effects of CAPS antibody on NE release.

Cytosolic CAPS Is Not Essential for Glutamate Release

In synaptosomes, ~37% of the total CAPS is membrane associated, while the remaining portion appears to be soluble in the cytoplasm (Berwin et al., 1998). To test whether the cytosol or the membrane was the source of CAPS in the assay and potentially played an as yet undetected role in glutamate release, antibodies were added either to the synaptosome preparation or to the cytosol prior to stimulation. When synaptosomes were exposed to the CAPS antibody and stimulated in the presence of untreated cytosol, neither glutamate nor [^{3}H]NE release was significantly different from the control values (Figure 4A). However, if untreated synaptosomes were stimulated with Ca^{2+} in the presence of CAPS antibody-treated cytosol, [^{3}H]NE release was reduced by nearly 50%, whereas glutamate release was unaltered, suggesting that at least half of the total NE release requires recruitment of cytosolic CAPS. This result rules out the possibility that the antibodies induced some nonspecific steric hindrance of the exocytotic machinery, since the antibodies did not need to act directly on synaptosomal membranes for inhibition.

To directly investigate a role for CAPS in NE but not glutamate release, cytosol immunodepleted of endogenous CAPS (Figure 4B, inset) was supplemented with recombinant CAPS. Although the CAPS-deficient cytosol supported glutamate release as effectively as control cytosol, it failed to reconstitute fully the [^{3}H]NE release. In the absence of CAPS, [^{3}H]NE release was reduced by nearly 50% of the control value. The decreased activity of the CAPS-deficient cytosol was restored fully by the addition of recombinant CAPS, reaching a value of 120% of the control value, suggesting that CAPS may be limiting in the assay. In contrast, the extent of glutamate release was not altered in the absence or presence of recombinant CAPS. Thus, CAPS is not essential for reconstitution of glutamate release, implying differential regulation of exocytosis for vesicle populations that store different neurotransmitters.

Discussion

The present study provides direct evidence of differential regulation of the molecular machinery that controls the secretion of two major neurotransmitters, NE and glutamate, in the mammalian brain. The ability now to identify a specific cytosolic factor involved in the Ca^{2+}-dependent release of NE but not glutamate suggests the existence of regulatory branch-points in the exocytotic pathways within the central nervous system and represents an important first step in analyzing, at the biochemical level, the molecular machinery directing vesicle recruitment, docking, and fusion at the synapse.

CAPS Does Not Regulate Glutamate Release from Mammalian Nerve Terminals

The most compelling evidence for the divergent control of glutamate release versus other transmitters was the ability of recombinant CAPS to promote NE release that had been reduced by immunodepletion of CAPS from cytosol. In contrast, glutamate secretion was fully reconstituted by CAPS-deficient cytosol and was unaffected by treatment with either recombinant CAPS or antibodies recognizing endogenous CAPS. The differential requirement of glutamate and NE release for CAPS implies that the synaptic vesicles that contain these transmitters undergo exocytosis, at least in part, via distinct molecular pathways. This interpretation is also consistent with the nearly 200-fold difference in Ca^{2+} sensitivity between [^{3}H]NE and glutamate release. For glutamate, the effective concentration range for the Ca^{2+} trigger was 50 μM to 1 mM with 250 μM Ca^{2+} provoking half-maximal transmitter release. This is similar to the estimated rise in the Ca^{2+} concentration in nerve terminals during action potentials and is close to the EC_{50} of the Ca^{2+} required to initiate exocytosis at central nerve terminals (Llinas et al., 1992; von Gersdorff and Matthews, 1994a; Heidelberger et al., 1994). In comparison, the sensitivity of [^{3}H]NE release to Ca^{2+} was significantly greater. Approximately 0.3 μM and 30 μM Ca^{2+} evoked threshold and maximal release, respectively, with half-maximal [^{3}H]NE release induced by 1 μM Ca^{2+}. This high sensitivity to Ca^{2+} for [^{3}H]NE release is comparable to that seen in other studies with synaptosomes (Dekker et al., 1991; Verhage et al., 1991; Hens et al. 1993a, 1993b) as well

as neuroendocrine cells (reviewed by Burgoyne and Morgan, 1995).

The high (~1 μM) and low (~200 μM) Ca^{2+} affinities described above are characteristic features of DCV and SV exocytosis, respectively (reviewed by Burgoyne and Morgan, 1995). These morphologically distinct vesicles are generally thought to store different neurotransmitters: SVs contain classical neurotransmitters such as glutamate, acetylcholine, glycine, and γ-aminobutyric acid (GABA) (reviewed by Calakos and Scheller, 1996), whereas large and small DCVs (LDCVs and SDCVs) store biogenic amines and/or peptide hormones (Thureson-Klein, 1983; Bauerfeind and Huttner, 1993; Goodall et al., 1997). Although in the vertebrate central nervous system the exclusive localization of catecholamines in DCVs remains uncertain, our results provide a strong argument that the vesicles that store NE and glutamate are functionally dissimilar. The observation by Berwin et al. (1998) that CAPS associates preferentially with DCVs rather than SVs supports this conclusion, along with other lines of evidence that indicate a divergence of the pathways that regulate DCV and SV exocytosis. Treatment with α-latrotoxin of the frog neuromuscular junction results in the depletion of SVs following exhaustive exocytosis, but no significant change in the DCV content (Matteoli et al., 1988). The coupling of stimulation frequency to DCV exocytosis (10–20 Hz) is substantially different from that of SVs (0–5 Hz) (Andersson et al., 1982; Ip and Zigmond, 1984; Weldon et al., 1993; reviewed by Collier, 1996). Morphological analyses show that DCVs docked at the plasma membrane are rarely encountered, whereas SVs are commonly clustered at or around active zones (Thureson-Klein, 1983; Verhage et al., 1991; Bruns and Jahn, 1995). Thus, the regulation by CAPS may represent just one aspect of the physiological disparities between SV and DCV exocytosis.

Protein sequence comparison suggests that CAPS is the mammalian homolog of the *C. elegans* UNC-31 protein, sharing 54% identity (Ann et al., 1997). Mutations in *unc-31* confer resistance to inhibitors of acetylcholinesterase, a phenotype indicative of impaired cholinergic neurotransmission, and promote serotonin accumulation (Desai et al., 1988; Miller et al., 1996). The latter result is consistent with a role for CAPS in DCV release. However, our results now raise the possibility that the reduced exocytosis of acetycholine-containing SVs in *unc-31* mutant worms may be indirect. Indeed, mutant worms are lethargic but not paralyzed (Avery et al., 1993), arguing that the machinery regulating acetylcholine release is functional. One possibility is that the inability to release serotonin negatively influences the activity of cholinergic neurons.

Contribution of Soluble and Membrane-Bound CAPS to Secretion

The reduced ability of the CAPS-deficient cytosol to support NE release suggests that at least half of the total NE stored in vesicles must proceed through a step that entails recruitment of CAPS from cytosol. Because nearly 40% of synaptosomal CAPS is membrane associated (Berwin et al., 1998), it is presumably this fraction that contributes to some of the remaining [^{3}H]NE release, as indicated by the difference between the maximal inhibition by the CAPS antibody (75%) and the activity of the CAPS-depleted cytosol (50%). It is possible that the contribution of the membrane-bound pool was underestimated in the experiments involving antibody treatment of synaptosome membranes because the antibody may have been subsequently neutralized by the exogenous CAPS provided in the cytosol or by recruitment of the exogenous CAPS to membranes. It is unlikely that any of the inhibition induced by the antibodies was the result of nonspecific steric hindrance since preimmune antibodies had no effect on transmitter release, and exocytosis could be inhibited by treating cytosol with CAPS antibodies when the exocytotic machinery in the synaptosomes was not directly exposed to the CAPS antibodies. Also, since glutamate and [^{3}H]NE exocytosis in these experiments was measured simultaneously, both might be expected to be similarly affected if the mode of inhibition was a nonspecific action on more general factors directing exocytosis. Finally, the specificity of the antibodies is demonstrated by the fact that recombinant CAPS completely reversed the activity of the immunodepleted cytosol.

The extent of Ca^{2+}-dependent [^{3}H]NE release in the presence of CAPS antibodies was close to the size of the cytosol-independent [^{3}H]NE pool. It is therefore reasonable to conclude that the role of CAPS in nerve terminals is carried out very late in the maturation in NE-containing vesicles, just prior to reaching a readily releasable state.

Differential Regulation of Vesicle Fusion in Semi-Intact Synaptosomes Reconstitutes the Events Observed In Vivo

The release of glutamate from semi-intact synaptosomes exhibited several phenotypic properties of exocytosis evident in vivo. The functionality of the Ca^{2+}-dependent trigger observed was preserved and specifically inhibited by clostridial neurotoxins. In addition, Ca^{2+}- and cytosol-dependent transmitter release from semi-intact synaptosomes was accompanied morphologically by a substantial decrease in the number of vesicle profiles. Given that glutamate is stored within rat cortical synaptic vesicles (Burger et al., 1989), it follows that these vesicles are the source of the observed Ca^{2+}-dependent release. The decrease in vesicle number is not an unexpected finding, assuming that endocytosis is unable to keep pace with exocytosis in vitro. Indeed, several studies indicate that the speed of vesicle recycling is inversely proportional to the duration of the intraterminal rise in Ca^{2+} (Ryan et al., 1993; von Gersdorff and Matthews, 1994b; Ryan and Smith, 1995). Consequently, the exposure to the Ca^{2+} trigger lasting many minutes in the present study, as opposed to transient spikes of millisecond duration that occur in vivo, may represent an obstacle to efficient membrane recycling.

Although cytosolic factors are essential for exocytosis from a variety of neuroendocrine cells (Holz et al., 1991; reviewed by Martin, 1994; Burgoyne and Morgan, 1995), their influence in central nerve terminals has not been examined until now. In the case of Ca^{2+}-dependent glutamate release, optimal reconstitution could potentially

involve one or more of the other cytoplasmic proteins that have been implicated in secretion (reviewed by Fischer von Mollard et al., 1994; Bean and Scheller, 1997) or potentially novel factors. Fusion of vesicles associated with membrane fragments derived from hypotonically lysed synaptosomes occurs without exogenous cytosol (Mehta et al., 1996). In the present study, the size of the Ca^{2+}-dependent, cytosol-independent pool comprised only 3% and 5% of the compartmentalized [^{3}H]NE and glutamate, respectively. This value matches the proportion of vesicles in close apposition to active zones in rodent cortical neurons (~3.7%; Schikorski and Stevens, 1997), raising the possibility that this transmitter pool is morphologically manifest as docked synaptic vesicles.

The additional 4- to 6-fold stimulation of transmitter release by cytosol indicates that the majority of releasable transmitter in nerve terminals must advance through various priming steps that are regulated, at least in part, by soluble cytoplasmic factors. This second, larger component of secretion is thereby defined as the reserve pool that undergoes priming reactions before arriving at a fusion-ready state. The morphological counterpart to this reserve pool is most likely the clusters of undocked synaptic vesicles in the cytoplasm of synaptosomes.

In conclusion, the in vitro approach that we have established to assess the role of cytosolic factors active in the targeting and fusion of synaptic vesicles has enabled us to demonstrate that glutamate-containing vesicles undergo priming and fusion independently of CAPS. These results suggest that biochemically and morphologically distinct neurotransmitter-containing vesicles progress to exocytosis via separate molecular pathways. Our experimental system now provides a biochemical approach with functional nerve terminals to explore interactions between protein components of the transport machinery that distinguish glutamate release from other neurotransmitters.

Experimental Procedures

Materials

SLO was initially purchased from Murex Diagnostics (Dartford, United Kingdom) and subsequently the recombinant form of the protein was obtained from Dr. S. Bhakdi (University of Mainz, Mainz, Germany). The activity of the recombinant SLO was easily calibrated and reproducible and is therefore recommended for the purposes of permeabilizing synaptosomal membranes.

Secretion from Permeabilized Synaptosomes

Synaptosomes were prepared as described (Fischer von Mollard et al., 1991). Briefly, male Sprague-Dawley rats (250 g) were decapitated following halothane-induced anesthesia, and the cerebral cortices were dissected and homogenized in ice-cold buffer A (320 mM sucrose, 1 mM EGTA, and 5 mM HEPES [pH 7.4]). The homogenate was centrifuged at 1,050 × g for 10 min. The supernatant was spun for 15 min at 13,300 × g and the resulting pellet (P2) resuspended in buffer A. The P2 fraction was loaded onto a discontinuous FICOLL gradient (13%, 9%, 5% in buffer A) and centrifuged for 35 min at 60,000 × g. The 13%–9% interface, containing intact synaptosomes, was resuspended in buffer B (140 mM NaCl, 5 mM KCl, 20 mM HEPES, 5 mM $NaHCO_3$, 1.2 mM Na_2HPO_4, 1 mM $MgCl_2$, 1 mM EGTA, and 10 mM glucose). In experiments measuring [^{3}H]NE release, intact synaptosomes were preincubated for 25 min at 25°C in buffer B with [^{3}H]NE (10 μCi/ml) before permeabilization.

Permeabilization was done according to the method of Bhakdi et al. (1993). The optimal SLO concentration for permeabilization was determined for each batch of the SLO purchased. SLO activity was carefully calibrated by measuring the efflux of the cytoplasmic marker lactate dehydrogenase (LDH; EC 1.1.1.27) determined according to the method of Worthington (1988). The optimal SLO concentration was the maximal concentration that caused no leakage of LDH or glutamate at 4°C. Briefly, SLO (6.0 μg/ml) was prebound to synaptosomes at 4°C for 2–3 min. This SLO concentration did not affect LDH release during exposure on ice. Residual free toxin was removed by washing with buffer B. Pore formation was subsequently initiated by incubating synaptosomes at 37°C for 3 min. LDH activity released during this step routinely reached ~60% of the total synaptosomal LDH activity, and this was not affected by the addition of Ca^{2+}.

Permeabilized synaptosomes (200–400 μg protein) were pelleted and resuspended in 1 mM EGTA in buffer C (145 mM KOAc, 25 mM HEPES, 2.5 $MgCl_2$, and 1 mM ATP [pH 7.2]) at 4°C for at least 15 min to allow for washout of soluble proteins. Synaptosomes were then resuspended in 50–200 μl buffer C supplemented with Ca^{2+} and cytosol, and transmitter release was initiated by increasing temperature to 25°C or 37°C. The reaction was stopped by the addition of cold 10 mM EGTA (final volume 1 ml) and centrifugation at 15,800 × g. The supernatant was assayed for glutamate or [^{3}H]NE.

Reagents to be tested for their effects on transmitter release were generally added after pore formation during the 15 min wash step and/or during the stimulation step. Prior to addition, the botulinum neurotoxins (donated by Dr. B. R. DasGupta, University of Wisconsin, Madison, WI) were treated with 1 mM DTT for 30 min at room temperature to separate the heavy and light chains.

Measurement of Transmitter Release

Glutamate was determined as described (Nicholls et al., 1987). Briefly, to the test sample were added (final concentration) 10 mM NADP, 1 mg/ml BSA (ultra fatty acid–free; Boehringer Mannheim), 40 IU glutamic dehydrogenase (EC 1.4.1.3; Sigma). The mixture was incubated at 37°C for 5 min and the fluorescence of NADPH determined with a Perkin Elmer LS50B luminescence spectrometer (excitation, 340 nm; emission, 460 nm).

Release of [^{3}H]NE into the supernatant was determined using liquid scintillation spectrometry.

Protein was determined with the Micro BCA Protein Assay (Pierce).

Preparation of Cytosol

Rat brains were thoroughly homogenized in 85 mM sucrose, 100 mM KOAc, 1 mM MgOAc, and 20 mM HEPES (pH 7.4). The homogenate was centrifuged for 10 min at 15,000 × g and the supernatant spun for 1 hr at 100,000 × g. The supernatant was subsequently dialyzed for 4 hr in 145 mM KOAc and 25 mM HEPES (pH 7.2) and frozen at −80°C.

CAPS was immunodepleted from crude cytosol as described previously by Walent et al. (1992) and replaced with recombinant CAPS protein that was expressed and purified according to Ann et al. (1997).

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